

EXHIBIT [A]

Example 8. Obtaining and functional testing of an anti-Gli3 scFv protein with Transportan at the C-terminal position

Recombinant anti-Gli3 scFv constructs composed of the VH and VL domains of a corresponding monoclonal antibody sequence (clone 5E1), connected via a flexible 19-residue linker, were cloned into pET40 vector using methods known in the art. In one construct, transportan was included in the fusion protein at its C-terminus. The proteins were expressed in *E. coli* in fusion with N-terminal bacterial chaperone DsbC and (His)₆-tag, and affinity-purified over Ni-NTA beads.

Below are the protein sequences of the obtained recombinant anti-Gli3 5E1 scFv constructs with and without Transportan. VH and VL domains appear in bold, linker residues in italics and Transportan sequence is underlined.

DsbC-5E1-Tra

SEQ ID NO:1

1	MKKGFMLFTL LAAFSGFAQA DDAAIQQTla KMGIKSSDIQ PAPVAGMKTv	50
51	LTNSGVLYIT DDGKHIIQGP MYDVSGTAPV NVTNKMllKQ LNALEKEMIV	100
101	YKAPQEKHVI TVFTDITCGY CHKLHEQMAD YNALGITVRY LAFPRQGLDS	150
151	DAEKEMKAIW CAKDKNKAFD DVMAGKSVAP ASCDvDIADH YALGVQLGVS	200
201	GTPAVVLSNG TLVPGYQPPK EMKEFLDEHQ KMTSGKGSTS GSGHHHHHHS	250
251	AGLVPRGSTA IGMKETAAK FERQHMDSPD LGTDDDDKSP GFSSTMAISD	300
301	PRVQLQQSGP ELVKPGASVK ISCKASGYSF TGYFMNwVKQ SHGKSLEWIG	350
351	RINPYNGDTF YNQKFKGKAT LTVDKSSSTA HmellSLTSE DSAVYYCGRS	400
401	GYDLYAMDYW GQGTSEFSSG GGGSGGGGSG GSVDQIVLTQ SPAIMSASPG	450
451	EKVTMTCSAS SSVSSRYLHW YQQKSGASPK LWIYGTSNLA SGVPARFSGS	500
501	SGTSSYSLTI SSVEAEDAAT YYCQYHSDP WTFGGGTKEF <u>GWTlnSAGYL</u>	550
551	<u>LGKINLKALA ALAKKIL</u>	

DsbC-5E1

SEQ ID NO:2

1	MKKGFMLFTL LAAFSGFAQA DDAAIQQTla KMGIKSSDIQ PAPVAGMKTv	50
51	LTNSGVLYIT DDGKHIIQGP MYDVSGTAPV NVTNKMllKQ LNALEKEMIV	100
101	YKAPQEKHVI TVFTDITCGY CHKLHEQMAD YNALGITVRY LAFPRQGLDS	150
151	DAEKEMKAIW CAKDKNKAFD DVMAGKSVAP ASCDvDIADH YALGVQLGVS	200
201	GTPAVVLSNG TLVPGYQPPK EMKEFLDEHQ KMTSGKGSTS GSGHHHHHHS	250
251	AGLVPRGSTA IGMKETAAK FERQHMDSPD LGTDDDDKSP GFSSTMAISD	300
301	PRVQLQQSGP ELVKPGASVK ISCKASGYSF TGYFMNwVKQ SHGKSLEWIG	350
351	RINPYNGDTF YNQKFKGKAT LTVDKSSSTA HmellSLTSE DSAVYYCGRS	400
401	GYDLYAMDYW GQGTSEFSSG GGGSGGGGSG GSVDQIVLTQ SPAIMSASPG	450

451	EKVTMTCSAS SSVSSRYLHW YQKSGASPK LWIYGTSNLA SGVPARFSGS	500
501	SGGTSYSLTI SSVEAEDAAT YYCQYHSDP WTFGGGTALA AALEHHHHHH	550
551	H	

The resulting anti-Gli3 fusion proteins were labelled with AlexaFluor 488 fluorescent dye and tested for their ability to enter into cultured Cos-7 cells. The proteins were applied for 30 minutes, the cells were washed and fixed, and fluorescence microscopy was performed. Figure 4A depicts internalisation of DsbC-5E1-Tra and Figure 4B depicts internalisation of DsbC-5E1. As can be seen, both proteins show very low internalisation efficiency.

In addition, internalisation properties of the above proteins were tested by flow cytometry. Cultured HEK-293 cells were incubated with the proteins for 2 hours, washed and analysed for intensity of fluorescent signal from the cell interior. The assay also included a control protein, DsbC-5E1 + TP10, comprising DsbC-5E1 that had been chemically conjugated to transportan TP10 peptide in a manner described in Example 3. The results are shown in Figure 4C (black = background fluorescence; red = DsbC-5E1-Tra; green = DsbC-5E1; blue = DsbC-5E1 + TP10). There was no difference in signal intensity between DsbC-5E1-Tra and DsbC-5E1, while DsbC-5E1 + TP10 showed markedly enhanced internalisation.

These findings suggest that the presence of Transportan *per se* does not guarantee efficient entry of anti-Gli3 scFv proteins into cells, and that positioning of the peptide is important in determining good protein internalisation properties. While in the TP10-conjugated variant the exact location of the peptide cannot be determined due to heterogeneity of the conjugated molecules, the position of Transportan in the DsbC-5E1-Tra fusion protein is clearly defined, yet the peptide remains inactive. Therefore one can conclude that creating a cell-penetrating anti-Gli3 scFv fusion protein with Transportan in a well-defined, active position is not trivial and requires a considerable amount of experimental work, which has not been previously undertaken. Thus it appears to a person skilled in the art that arriving at the present invention is not obvious or easily derived from prior knowledge, and is therefore considered to involve an inventive step.

Example 9. Target binding by an anti-Gli3 scFv protein with Transportan at the C-terminal position

To determine whether inclusion of Transportan in a recombinant anti-Gli3 scFv fusion protein influences binding of the scFv to its target protein, ELISA assays were carried out with DsbC-5E1-Tra and DsbC-5E1 created against human Gli3 (see Example 8). Anti-Gli3 monoclonal antibody clone 5E1, the parent antibody from which the scFv constructs were derived, was used as a positive control. 96-well ELISA plates were coated with human Gli3 protein at 30 µg/ml, washed and blocked with bovine serum albumin. Anti-Gli3 scFv proteins or MAb were then added in solution at concentrations ranging from picomolar to micromolar, and the plates were incubated for 1 hour at room temperature. After washing, bound proteins were detected using an anti-His-tag or anti-mouse IgG antibody conjugated to horse radish peroxidase. Signal was

generated using OPD as a substrate, and optical density resulting from the ensuing colour reaction was quantified using an ELISA plate reader. Values for specific binding to Gli3 were obtained by subtracting background values (binding to albumin-blocked wells not containing Gli3) from total binding.

The resulting normalised dose-response curves from three independent assays are presented in Figure 5, which shows that while both DsbC-5E1-Tra and DsbC-5E1 bind to their target with a somewhat lower efficiency than the control MAb 5E1, there is no substantial difference in affinity between the two recombinant proteins. From this finding one can conclude that inclusion of Transportan at the C-terminus of the fusion protein DsbC-5E1-Tra does not interfere with the protein's capability to recognise its target Gli3.

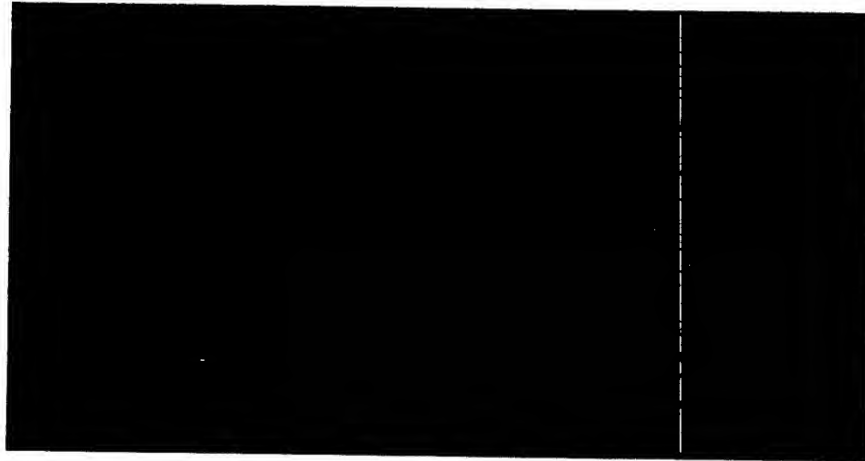


FIGURE 4A

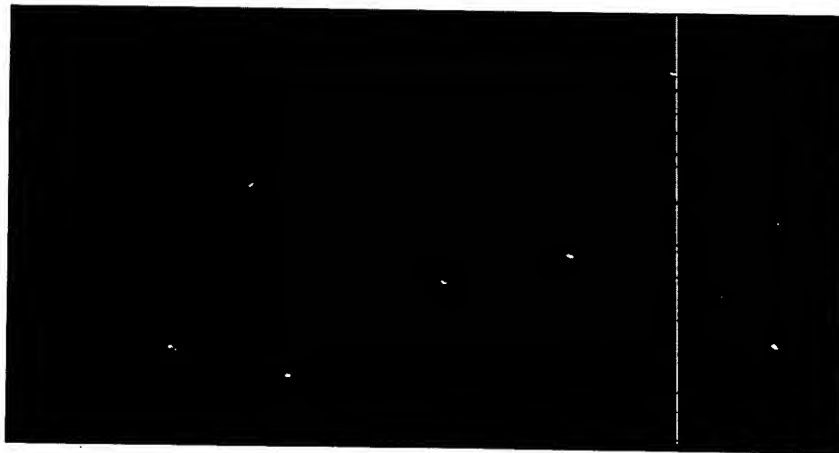


FIGURE 4B

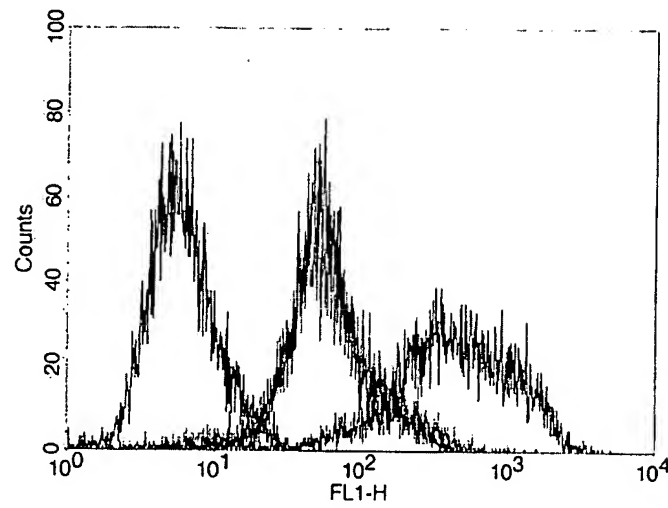


FIGURE 4C

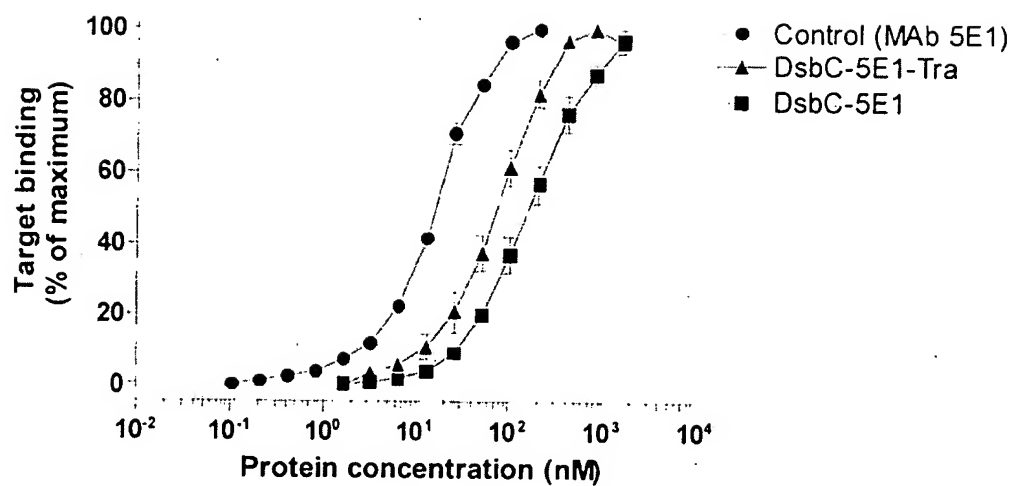


FIGURE 5